Fully Integrated downstream process to enable Next Generation Manufacturing

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Abstract

Next generation manufacturing (NGM) has evolved over the past decade to a point where large biopharmaceutical organizations are making large investments in the technology and considering implementation in clinical and commercial processes. There are many well-considered reasons to implement NGM. For the most part, organizations will not fund NGM unless the implementation benefits the funding organization by providing reduced costs, reduced time or additional needed capabilities. Productivity improvements gained from continuous purification are shown in this work, which used a new system that fully integrates and automates several downstream unit operations of a biopharmaceutical process to provide flexibility and easy implementation of NGM. The equipment and automation supporting NGM can be complicated and expensive. Biopharmaceutical Process Development considered two options: (1) design its own NGM system or (2) buy a pre-built system. PAK BioSolutions (Virginia, US), provides a turn-key automated and integrated system that can operate up to four continuous purification stages simultaneously, while maintaining a small footprint in the manufacturing plant. The PAK system provides significant cost benefits (˜10x lower) compared to the alternative – integration of many different pieces of equipment through a Distributed Control System (DCS) that would require significant engineering time for design, automation and integration. Integrated and Continuous Biomanufacturing can lead to significant reductions in facility size, reduced manufacturing costs, and enhanced product quality when compared to the traditional batch mode of operation. The PAK system uses new automation strategies that robustly link unit operations. We present the optimized process fit, sterility and bioburden control strategy, and automation features (such as pH feedback control and in-line detergent addition) that enabled continuous operation of a 14 day end-to-end monoclonal antibody purification process at the clinical manufacturing scale.

Introduction

Continuous manufacturing processes have gained significant interest from biopharmaceutical companies in recent years due to their potential to provide large productivity increases over traditional batch production modes (Konstantinov & Cooney, 2014; Pollock, 2017; Arnold 2018). These productivity increases allow a company to produce more biopharmaceutical product from an existing manufacturing facility or build new facilities with significantly reduced footprint compared to traditional designs.

Progress on upstream continuous processes has been widely published and presented at industry conferences. Companies have reported titers for perfusion processes reaching 2.3 g/L per day and beyond at scales over 500L (Coffman, 2021). This higher cell culture productivity results in a purification bottleneck for traditional batch purification processes. Perfusion cell culture volumes are typically on the order of 1.5 cell culture vessel

volume per day over 30 days and have viable cell densities (VCD) and titers that vary over 10X across this duration. A traditional 4 x 12,000L facility can now be replaced with a 5 x 2,000L facility that outputs the same product mass, but at a cost of over 6X more cell culture volume that must be forward processed (Arnold, 2018). Purification facilities are not equipped with vessels to store the entire batch volume and therefore, at a minimum, must process the material through the purification unit operations in multiple sub-batches.

Continuous purification unit operations have matured in recent years and technologies now allow a full end-to-end continuous process without the need for large hold vessels (Pezzini, 2018). These technologies eliminate the challenge previously presented by large perfusion harvest volumes. In a continuous purification process, each unit operation in the process is connected in series and operated simultaneously. Since the material is continuously forward processed from one step to the next, there is no need to hold large intermediate volumes in tanks. Furthermore, the purification process keeps pace with the perfusion bioreactor and the full batch of drug substance is purified within 12 hours of completion of the cell culture operation (Coolbaugh, 2021).

Productivity improvements gained from continuous purification extend beyond those achieved with perfusion cell culture processes (Klutz, 2015). Regardless of cell culture method, continuous purification offers benefits of reduced equipment size and footprint, reduced capital and consumable costs, and decreased run duration. Facility footprint is significantly reduced by elimination of product hold tanks and the need for utilities to clean and sterilize stainless steel piping systems. Low flow rates allow for smaller piping diameters that can be provided by single use tubing instead of stainless-steel piping. Advanced automation allows processes to operate all steps 24/7 without intervention compared to one step performed per shift, greatly improving utilization of existing facility and personnel resources. Smaller chromatography columns are cycled continuously throughout a run, reaching more than 35 cycles compared to 3 to 5 cycles on a larger column for a traditional batch process. As a result, resin utilization is maximized and resin costs are reduced (Otez, 2020). Although the capture chromatography step sees the most consumable cost reduction in a continuous purification process, labor and time savings for an end-to-end continuous process are notable (Mahal, 2021).

End-to-end continuous purification processes have been published previously by our group and others (Godawat, 2015; Pezzini, 2018, Coolbaugh, 2021). They include processes up to 25 days in duration and a demonstration of bioburden control. Even so, significant opportunities for process maturity exist for certain unit operations, equipment hardware and automation. Here, we present the latest generation of continuous purification processes with an off-the-shelf Pilot PAK continuous purification system (PAK BioSolutions, Virginia, US) that includes single-use GMP flow kits, advanced control strategies, and supervisory control and data acquisition (SCADA).

In this work, we demonstrate a 14-day continuous monoclonal antibody (mAb) purification process for a 50L perfusion bioreactor. Bioburden was controlled within specifications for the duration of the process. A single PAK automated system with four flow kits installed was used to perform the following steps; harvested perfusion material was processed through a Protein A capture chromatography step, a low pH virus inactivation step, a depth filter, a 0.2μm sterile filter and an anion exchange membrane. The process performed did not require a cation exchange chromatography step to meet process impurity specifications. Ultrafiltration/ diafiltration operations were performed separately after breaking down and setting up the same PAK system in a UFDF setup.

As demonstrated in this work, continuous cell culture and purification processes have advanced beyond proof-of-concept studies. The significant advancements in off-the-shelf equipment now enable routine operation of continuous purification processes in pilot and GMP facilities. This equipment reduces footprint requirements for an end-to-end process by 75% and offers a level of automation that reduces hands on time by 90% compared to batch. Furthermore, we present low pH viral clearance data required to implement a continuous purification process for a GMP facility.

Materials

Equipment and Consumables

A Pilot PAK end-to-end continuous purification system from PAK BioSolutions (Virginia, US) was used to perform all purification process steps. Along with the Pilot PAK system were four Pilot PAK flow kits from PAK BioSolutions (Virginia, US) that were used to link unit operations together. MabSelect PrismA® and Sephadex G-25 Coarse® size exclusion chromatographic (SEC) resins were purchased from Cytiva (Massachusetts, US). The three chromatography column housing units were Vantage L Laboratory Column VL 44 x 250 from MilliporeSigma (Massachusetts, US). The Opticap Sterile 0.2µm filter and the Mustang Q Membrane Capsule were purchased from MilliporeSigma (Massachusetts, US) and Pall (New York, US) respectively. Tryptic Soy Agar plates were purchased from VWR (Pennsylvania, US).

Buffers and Solutions

The buffer system utilized for dual-column capture chromatography (MabSelect PrismA®) included six buffers: (1) 50 mM Tris, pH 7.4; (2) 50mM Tris, 0.5 M NaCl, pH 7.4; (3) 25mM sodium acetate, pH 3.6; (4) 100mM acetic acid (5) 1N NaOH and (6) 2% benzyl alcohol, 100 mM sodium acetate, pH 5.0. For the Viral Inactivation (VI) step, the solution system utilized 1M acetic acid and 1M tris base. All buffers were tested for bioburden and only qualified for use with negative results.

Protein

The monoclonal antibody (mAb1) used in the continuous process studies was a humanized IgG1 produced from Chinese hamster ovary (CHO) cells by AstraZeneca. This protein has a molecular weight of 146 kDa, as determined experimentally by mass spectroscopy and has a theoretical isoelectric point of 7.15, as determined by amino acid sequencing.

The monoclonal antibody (mAb2) used in viral clearance studies was a humanized IgG1 produced from Chinese hamster ovary (CHO) cells by AstraZeneca. The mAb2 intermediate (17 mg/mL, pH 4.5) purified by protein A chromatography was used in the virus inactivation study as starting material.

Methods

Process overview

The purification process for this study included an automated and integrated dual-column chromatography step (DCC) (Angarita, 2015), leading into a continuous virus inactivation (VI) step and a final flow-through polishing AEX step. Upstream and downstream of the DCC step were 10L glass vessels to accommodate purification of 50L of cell culture material per day. The process flow path, the connections between each unit operation in the process and the automation to control and acquire the data from all unit operations in parallel, were executed with four 500mL flow kits and the Pilot PAK system from PAK BioSolutions (Virginia, US). The flow path of the process and the PAK flow kits are shown in Figure 1 and Figure 2, respectively. The 50L per day of harvested material was combined from several different perfusion bioreactors that had run prior to this study. The bags were chosen and connected in order to simulate the titer profile of a typical perfusion bioreactor process.

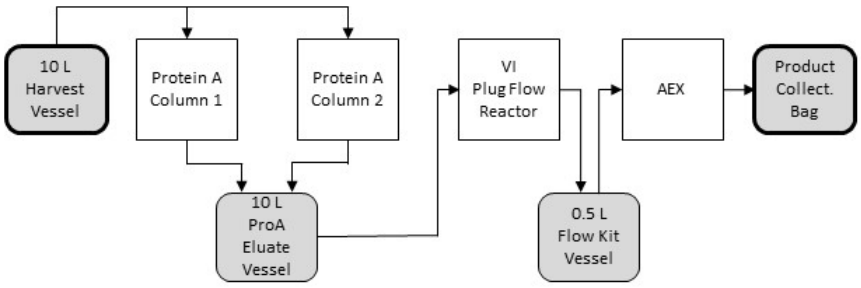


Figure 1. Process flow path in PAK System.

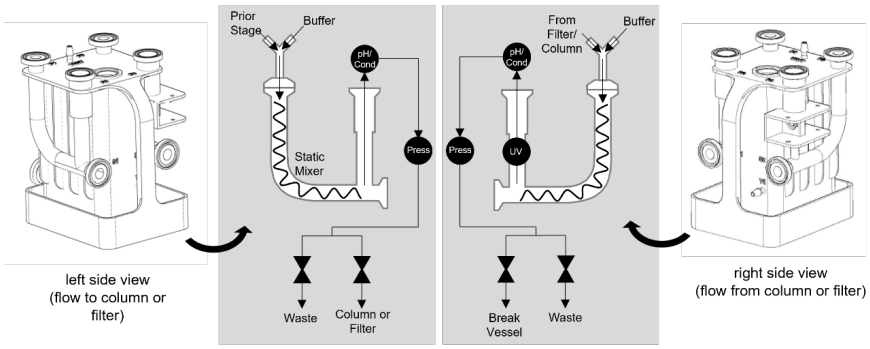


Figure 2. Flow path of the PAK single-use flow kit for individual process steps (chromatography, virus inactivation, membrane, filtration). Includes pH, conductivity (Cond), and pressure (P) instrumentation.

The 500mL flow kits are 3D-printed and include sensors (pH, conductivity, UV280nm, UV300nm, pressure), in-line mixers for buffer addition (pH titration, conductivity adjustment, in-line dilution), a vented mixing vessel and a sample port (Figure 2).

Sterilization and Sanitization Pre-Run

Bioburden control of the continuous purification process required a combination of sanitization and sterilization methods. The Pilot PAK flow kits and associated tubing assemblies were provided gamma-irradiated. Chromatography columns (MabSelect PrismA® used for capture step and SEC column used for VI step) were sanitized with 1N sodium hydroxide during automated start-up sequences, along with the associated flow paths that included pH, conductivity and UV instrumentation. The 0.2µm sterile filter and the Mustang Q membrane were sanitized offline per the vendor specifications with 1N sodium hydroxide prior to use. Glass vessels used as product collection tanks, situated after the perfusion bioreactor and capture step, were autoclaved with tubing. Consumable components such as bags and tubing were gamma irradiated or autoclaved. Sanitary quick connects were used to make any connections that were not sanitized prior to use.

Cell Culture

Several 3L bioreactors were used to generate recombinant IgG via a Chinese Hamster Ovary (CHO) cell line. These individual 3L bioreactors were harvested using coupled Tangential Flow Filtration (TFF), from which

they were combined into 50 and 100L bags according to their titers. The titer profile for the 14-day study presented here was generated by ordering the bags based on their titer to represent a typical 50L perfusion bioreactor process. These bags were then welded to the flow path and pumped at a rate of 50L/day into a 10L glass vessel before the dual-column chromatography step. The titer of the material used ranged from 0.2 – 2.34 g/L and totaled 490g of harvested mAb.

Dual Column Chromatography for Protein A Capture

The target molecule was captured with two 4.4 cm diameter x 7.7 cm height chromatography columns using MabSelect PrismA® resin at a capacity of 60 g/L. The columns for the capture step were operated using a dual-column strategy which operated in a typical bind and elute methodology, enabling a continuous load and discontinuous product elution. The size of the columns is optimized for protein mass and process time, resulting in higher resin utilization. Parallel column operation maintains one of the two columns in the load phase at all times. While one column is in the load phase, the other column cycles through the process equilibration, wash, elution, strip and sanitization buffers (Figure 3). The columns were loaded to 60 g/L every cycle; the load duration and frequency of elution varied with the change in titer (load volume was manually input into the PAK automation control screen daily). The elution collection criteria was specified as 5 column volumes (CVs), and collection began after the completion of the first CV. The eluate was collected in a 10L glass vessel downstream of the two columns.

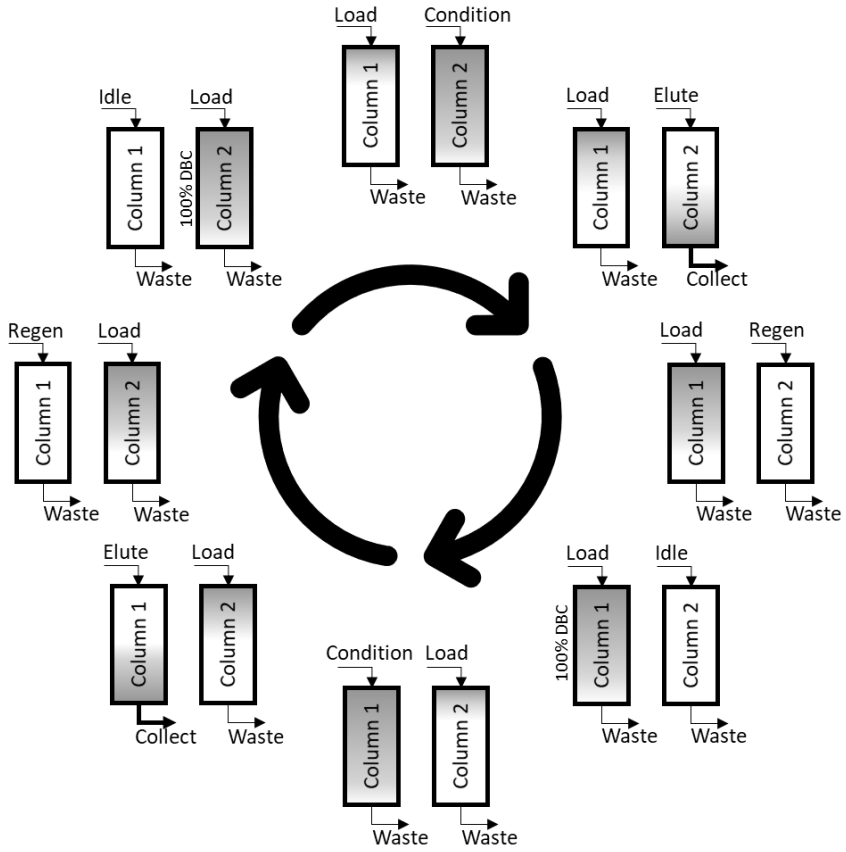


Figure 3. Diagram illustrating the dual column operation. Condition indicates pre elution phases such as equilibration and wash. Regen indicates the combination of the post-elution phases: strip, sanitization and

re-equilibration. DBC stands for dynamic binding capacity. Grey shade illustrates protein loaded into the column.

Virus Inactivation

The viral inactivation strategy implemented in this study used a plug flow reactor made from a 4.4 cm diameter x 20 cm height SEC (Size Exclusion Chromatography) column with Sephadex G-25 Coarse® resin to achieve a target residence time of 15 minutes. Acidification was performed before the SEC column and neutralization was performed post column. The capture product was titrated to an acidification target of pH 3.5 with an allowable range of ± 0.1. Acid was added in-line with the product line and the acidified product immediately flowed through the flow kit's static mixer and pH probe. A feedback PID controller is used to continuously adjust the acid pump flow rate to ensure the pH is within the desired range. After the pH is within the acidification target range for an appropriate amount of time, the product is directed through the SEC column which was equilibrated at pH 3.5. Post SEC column, an in-line addition of base is continuously fed to neutralize the acidic product to a target of pH 7.2 with an allowable range of ± 0.2. The viral inactivation procedure during this process began on day 8 of the 14-day run. The start day for this step is determined based on (1) product quality impact from hold time at capture product conditions and (2) system flow rate lower limit to allow VI to start and run for extended duration. For this study VI was run in three sub-batches. For a future optimized process, we aim to operate without pausing.

Anion Exchange Chromatography

The product from VI step flowed through a filter train comprised of two Opticap® Sterile 0.2µm filters on either side of a Mustang Q® Membrane Capsule. This filter train was prepped offline, prior to its connection to the system according to the vendor specifications. This prep work was done the day before the filter train was connected to the system (day 7). The filter train was connected on day 8; this timeline coincided with the start of the viral inactivation step. From here, material was collected in a final 50L product collection bag. This final polishing chromatography step was performed with a single-use membrane in a flowthrough mode. The membrane was not reused. The 0.2µm filter downstream of the Mustang Q membrane ensured that no potential bioburden reached the final product collection bag.

Bioburden Sampling and Testing Strategy

Samples were taken from four points in the process: (1) the cell culture harvest 10L vessel, (2) Protein A product 10L vessel, (3) VI product flow kit vessel and (4) final product collection bag. Samples were taken from sample ports that were specifically installed during set-up and were clamped closed when not in use to prevent potential exposure to the environment. Samples were taken aseptically using 10 mL syringes, about 24 hours apart. Samples were aliquoted into individual, labelled tubes under a biosafety cabinet in order to preserve sterility. Once in the tubes, the samples were stored at 2-8°C until they were ready to be submitted for analysis (see section Analytical Testing

for more details). This sampling method was repeated every day of the run, for each sampling point. The protein A bindable testing was done in order to record the daily titer at each stage. The HCP and DNA concentration assays tested the impurity level at each stage and therefore see how effective each unit operation was at removing the impurities. The SE-HPLC testing was done to determine the monomer purity at each stage and therefore determine daily product quality across the process. The samples for testing bioburden were taken and plated onto culture plates under a biosafety cabinet using L-shaped spreaders. Once done, the plates were stored inside of an incubation chamber running at 35 °C. The plates were monitored every day until 72 hours had passed in order to ensure the sterility of the system. These sampling and bioburden plating methods were repeated every day for each sampling point, through the end of the run.

Analytical Testing

Size exclusion-high performance liquid chromatography (SE-HPLC)

SE-HPLC was performed using a Waters 515 pump, a Waters 2487 Dual Absorbance Detector (Waters) and a Rheodyme 77,251 injector and a TSK Gel G3000 SWXL column (300 mm × 7.8 mm, Tosoh Biosep). The volume of injection was adjusted according to each sample concentration in order to inject 250 µg of mAb1, and separation was performed at a flow rate of 1.0 mL/min. The running buffer was composed of 100mM sodium sulfate, 100mM sodium phosphate dibasic, pH 6.8. UV detection was performed at 280 nm. The results were reported as monomer peak percentage and high molecular weight (HMW) content percentage of the total integrated area.

CHO DNA Real-Time PCR Assay

The assay is performed in a 96-well plate format using a forward and reverse primer set specific for the Chinese hamster short interspersed repetitive element (SINE) DNA sequence. A unique 64 base-pair product is generated from CHO genomic DNA calibrators and test samples within 40 cycles of the PCR. DNA is extracted from test samples and an extracted control using the DNA Extractor Kit® from Wako. Test samples, CHO genomic DNA calibrators and controls are added to a reagent master mix containing primers and SYBR Green. Cycling is performed in three steps as follows: (1) 10 minutes at 95°C for polymerase activation; (2) 40 cycles of 15 seconds at 95°C for denaturation; (3) 40 cycles of 60 seconds at 60°C for annealing of primers and extension. Quantities of DNA in the samples above LLOQ are regressed from an assay specific standard curve generated by the Sequence Detection Systems (SDS) software.

Gyros CHO Host Cell Protein (HCP) Assay

Standard curve, samples and controls are diluted in a proprietary Gyros buffer and loaded into sample microplates. In-house prepared biotinylated sheep anti-CHO HCP capture antibody and fluorescent labeled sheep anti-CHO HCP detection antibodies are diluted and loaded into a reagent microplate. The microplates are loaded on the Gyrolab instrument and automated execution of the assay is initiated. Once all reactions are complete in the CDs are read by the instrument using a fluorescence detector at 647 nm. HCP quantities are regressed from the standard curve generated by the software and corrected for dilution factor. Reported sample concentrations are the mean concentration of the dilutions yielding spike recovery values of 75-125%.

HPLC Protein A bindable Assay for mAb concentration

Protein A bindable HPLC is used to determine IgG concentration utilizing Protein A affinity chromatography separation technique. Test sample is injected onto a PA Immuno-Detection Sensor Cartridge (2.1 mmD x 30 mmL) with PBS buffer as a mobile phase. The sample flows through the cartridge where the target IgG specifically binds to the Protein A immobilized on the cartridge and non-target components are washed out. The bound IgG is eluted with 0.1% phosphoric acid in PBS, and detected using UV absorbance at 280 nm. The IgG content is then quantified using linear regression analysis of the standard curve prepared from the corresponding calibration standards. Results are reported in µg/mL.

Results and Discussion

This study successfully demonstrated a next-generation continuous purification process, running for 14-days at a rate of 50L of cell culture harvest material per day. Bioburden control was maintained for the duration of the run and product quality results were within acceptable limits. Advanced automation enabled tight control of the process to meet specifications. Automation control strategies also enabled break vessel volumes between 150 ml and 10L (20-fold or greater reduction compared to batch). Facility footprint of the process was reduced by 75% compared to the batch process, primarily due to the reduction in vessel volumes and ability to perform all three process steps in a single system. Setup time was to 1.5 days and was followed by 14 days of hands-free operation with the exception of daily sampling (90% reduction in operator hours).

These outcomes demonstrate the robustness of this commercially available automation system for continuous purification processes.

Bioburden Control

Most downstream operations for monoclonal antibody are not considered aseptic and operate as low-bioburden. The guidance commonly used sets a limit at 10 colony-forming units (CFU) per mL.

Bioburden control was demonstrated for the duration of the 14-day run. Samples were taken daily from the harvest break vessel, protein A product vessel, VI neutralized product vessel and AEX final product. None of the sampling points tested positive for bioburden except, on day 7, when 1CFU/mL was observed for the protein A product vessel (below the acceptable criteria of <10CFU/mL). This hit was attributed to intentionally opening the protein A product vessel to fix an incorrectly installed tubing assembly on the vessel. The bioburden objectives for this process were clearly met. The 1CFU/mL sample from the protein A elution product vessel was cleared by implementing a bioburden mitigation strategy. This strategy consisted of six important steps: (1) pausing the protein A step, (2) removing the contaminated vessel from the system, (3) autoclaving a new vessel, (4) filtering material into the new vessel under aseptic conditions, (5) integrating the new vessel into the process and (6) flushing lines with sanitization buffer before restarting the protein A step. No bioburden was detected after the implementation of this strategy. Successful implementation of this mitigation strategy demonstrates the ability to clear bioburden from an ongoing process without significant process disruption, a necessity to save a batch when operating a continuous operation.

Capture

Seventy protein A capture chromatography cycles were successfully run during the 14-day process. The protein A capture step began automatically once the harvest vessel reached a setpoint volume of 2L. The capture step operated for the duration of the run.

The capture step was operated under a constant mass load principle, targeting the resin capacity of 60g of mAb per L of resin: the load volume onto the column changed as the titer varied throughout the run to ensure that the grams of mAb loaded was the same for each cycle, as illustrated in Figure 4. Load volume decreases as titers increase until the middle of the run. Load volume then increases again as the titer drops towards the end of the run. This strategy ensures a constant concentration downstream of the protein A capture step, which greatly simplifies the downstream process dynamics. The standard deviation for the Protein A concentration over the duration of the run was 1.4 g/L. With constant downstream concentration, the virus inactivation step does not need to account for large mAb concentration changes that can affect the titration profile. Additionally, polishing chromatography step throughput can be calculated from volumetric throughput and the known concentration. The capture step was triggered to stop once the harvest vessel dropped to a low level due to lack of flow from the cell culture feed.

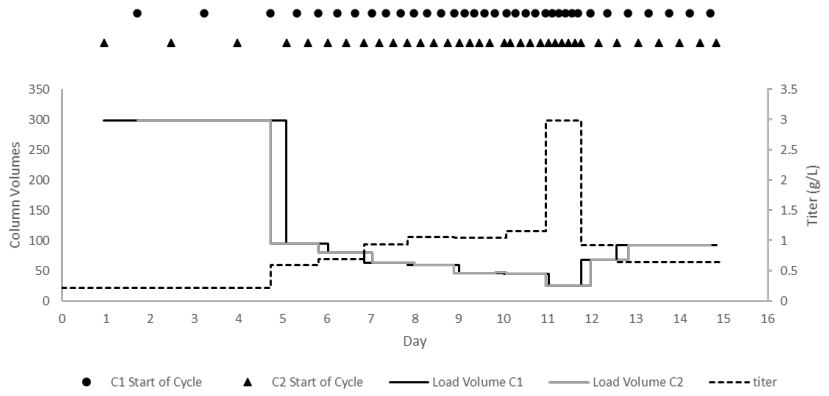


Figure 4. Protein A load volume and titer for the duration of the run. Black circles and triangles represent the start of the cycles for column 1 and 2, respectively.

Chromatogram overlays of the 35 cycles performed on each of the two capture columns were generated for analysis (data not shown). Constant peak height and position are observed across all cycles. Tailing was noticed on some cycles, especially towards the end of the run. Analysis of conductivity traces showed a change in transition curve profile for these cycles which indicates a change in bed structure rather than a change in impurity profile or issue with the UV meter. The root cause was likely air on the columns. This can easily be mitigated for future processes by installing a bubble trap prior to the protein A step, which was not in place during this run.

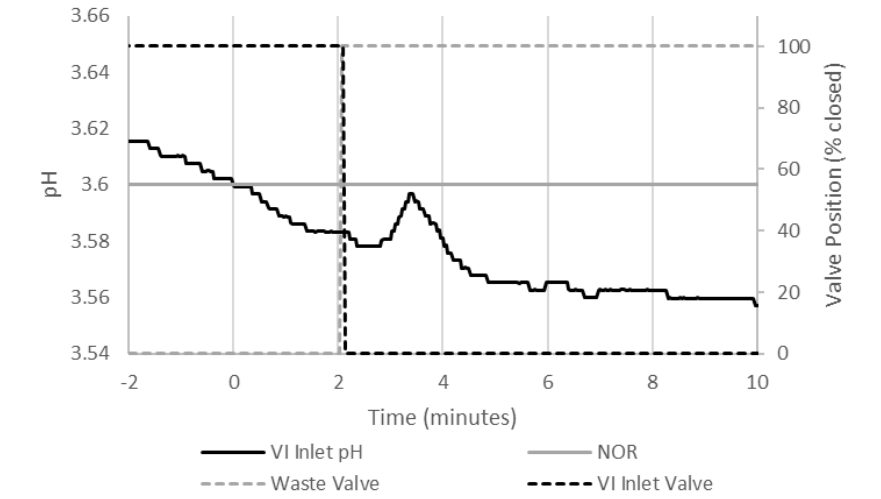
Virus Inactivation

The continuous low pH virus inactivation step was successfully controlled to meet pH and residence time specifications. The virus inactivation step was performed in 3 parts by starting and pausing this step 3 times during the 14-day process. This pausing was required to account for the change in rate of product elutions from the upstream protein A step and maintain a constant residence time across the virus inactivation column. The virus inactivation step was triggered to automatically start when the protein A product vessel of 10L filled to a high target volume. During the run, the virus inactivation step drained the vessel until it reached a low target volume, at which point the virus inactivation step automatically paused until the high target level was reached again. Each of the three virus inactivation sub-runs lasted approximately 1 day.

The virus inactivation step used an SEC column to provide the required inactivation residence time. The SEC column models a plug flow reactor and this choice benefits from the abundance of industry knowledge around column packing and qualification. Additionally, residence time distribution is independent of flow rate in a packed bed with no liquid-solid mass transfer, unlike tubular flow reactor designs which rely on dean vortices for mixing (Amarikwa, 2019). Other options, such as alternating tank VI were not considered due to the increased space, equipment, and automation requirements.

The pH of the capture product was titrated inline down to a pH of 3.5 with 1 M of acetic acid, before passing through the SEC column. After exiting the SEC column, pH was titrated inline up to a target pH of 7.2 with 1M tris base. Inline pH adjustments were performed using feedback control. The pH was well controlled for and was mostly maintained within the specified ranges of ± 0.1 and ± 0.2 for the acidification and neutralization, respectively. Any disturbances outside of the specified ranges were diverted to waste until pH returned within spec and remained stable for 2 minutes. pH data can be seen in Figure 5.

a



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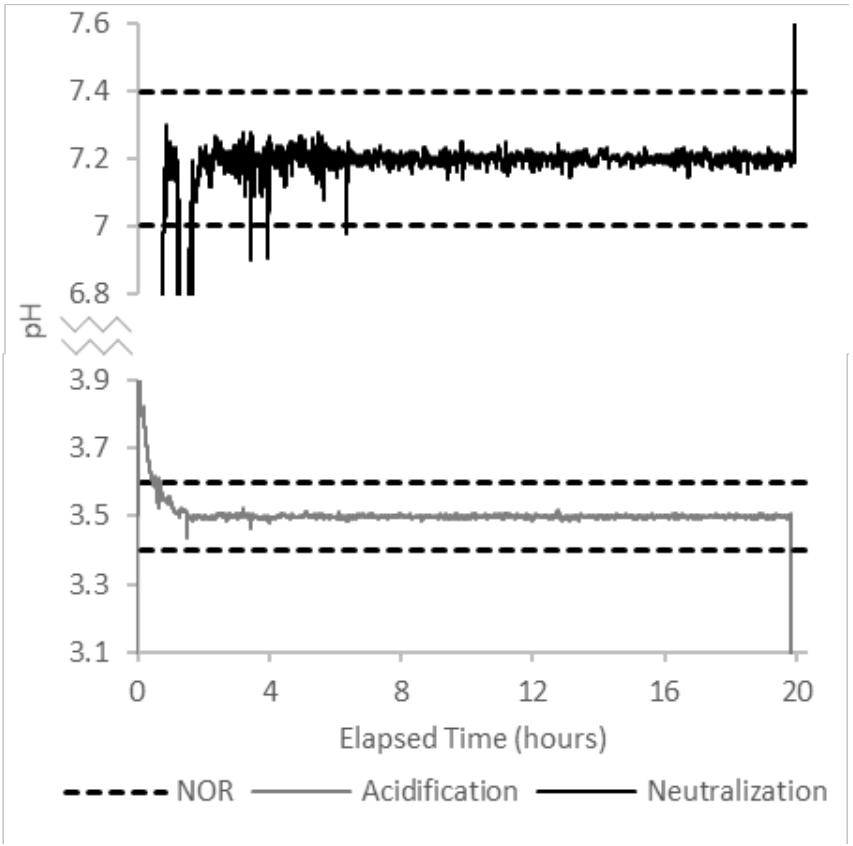

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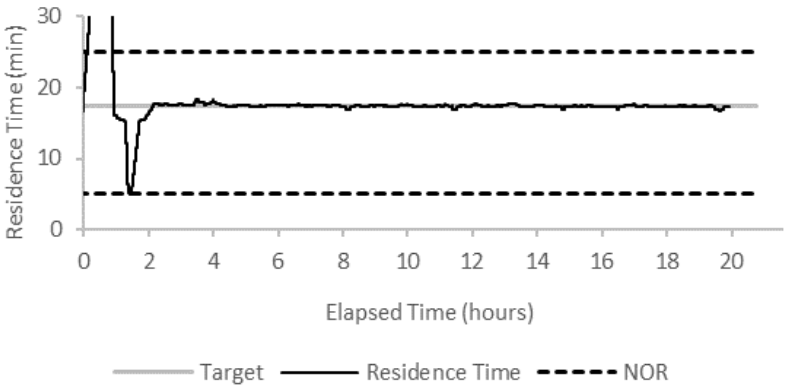


Figure 5. Virus inactivation pH trends. (a) Valve switch to in-line with process during startup. (b) pH trends for sub-run 3. NOR is normal operating range defined by the process description. (c) Virus inactivation residence time trend during sub-run 3.

Residence time through the SEC column was also controlled to a setpoint through feedback control. Rather than simply setting the pump to a fixed flow rate to achieve the required residence time, the last pump in the process changed flow rate to drive the overall mass flow rate of the process to match the flow rate required to achieve the target residence time in the SEC column. This strategy is required when running a truly integrated process as fixed flow rates on any step will inevitably result in vessel over/underflow events due to imperfectly calibrated pumps.

Polishing

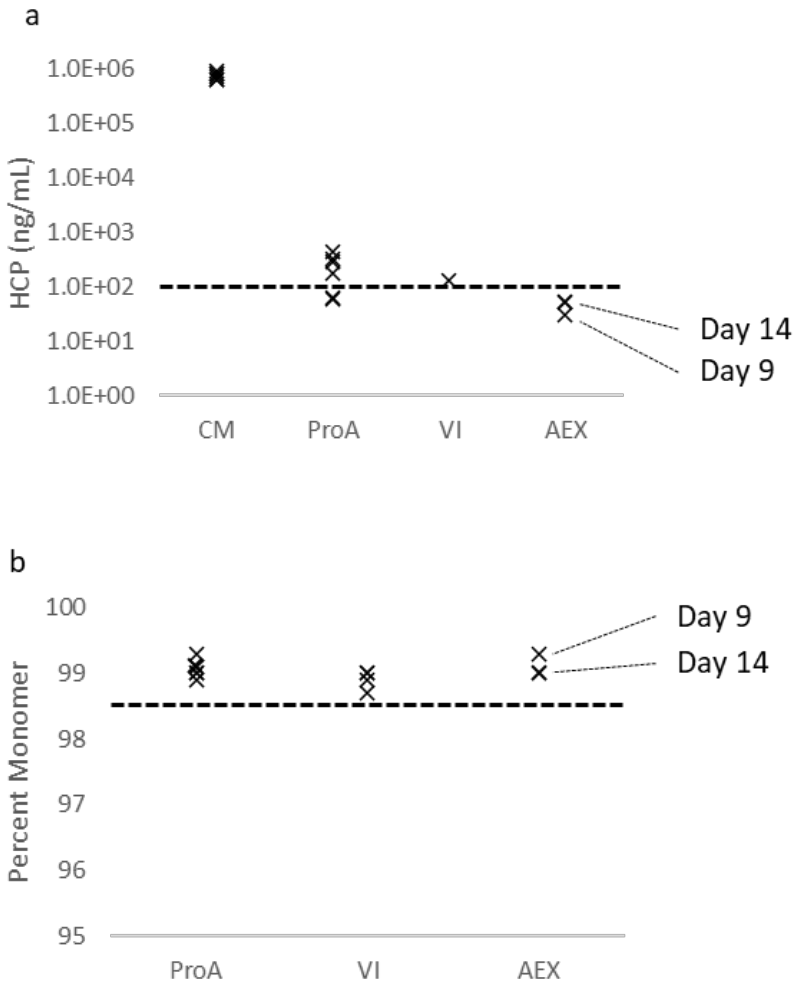
A flow-through anion exchange membrane polishing step was performed for this run. A second polishing step was not required to meet product quality specifications. A 0.2μm filter was installed before and after the Pall Mustang Q AEX membrane, which was immediately prior to the final collection bag. The filter train, which consisted of the AEX membrane and two 0.2μm filters (pre and post membrane), was swapped with a fresh filter train once the capacity on either the filter or membrane had been reached. The filters were sized in order to match their capacity with the AEX membrane and were replaced at the same time. The throughput was automatically determined by the PAK system, which alerted the user when a swap was required.

The filter train was swapped once during the process, although ideally, two swaps would have been performed. Unfortunately, the COVID-pandemic impacted global supplies and limited the availability of single-use materials like filters and membranes, thus only one swap was able to be performed. This resulted in an intentional overloading of the second Mustang Q to prevent cutting the run short. The first train was loaded to 14.2 liters while the second was loaded to 30.4 liters. Aside from the consequence of limited membrane area, the polishing step ran as expected.

Product Quality

The continuous purification process performed as expected and demonstrated acceptable product quality results. DNA, HCP, and monomer purity measurements were performed. Samples were taken from the harvest vessel, protein A product vessel, virus inactivation product flow kit, and the final collection bag. The final collection bag contained all the material collected from a single filter train. Product was diverted to a new collection bag when the filter train was swapped. The final AEX product was within acceptable ranges for percent monomer and host cell protein on both days measured. DNA levels were acceptable on

day 9 but out of range on day 14. This was attributed to the intentional overloading of the second AEX membrane as described above. Overall, product quality was comparable to the batch downstream process, with the exception of the day 14 AEX DNA datapoint. Results can be seen in Figure 6.



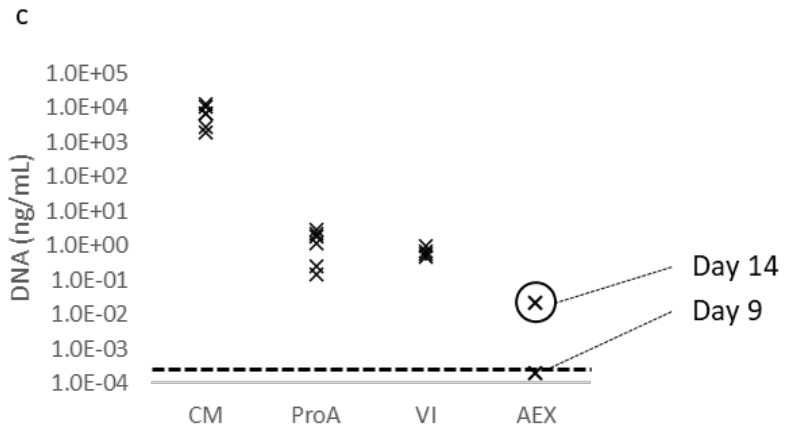


Figure 6. Product quality results from each sampling point across the duration of the run. Black dashed line indicated the acceptable cut-off for the final AEX product. Each datapoint is from a different day in the process. AEX final product sample points are labeled with day of sample collection. (a) HCP titers in ng/mL (acceptable cut-off below line). (b) Percent monomer (acceptable cut-off above line). (c) DNA titers in ng/mL (acceptable cut-off below line). Circled DNA datapoint on AEX step shows out of spec sample due to intentional overloading of AEX membrane.

Advanced Automation Control Strategies

A key objective of this work was to evaluate the ease of integrating multiple unit operations, which requires complex automation strategies. Unit operation integration was handled completely by the PAK system, which removed the burden of in-house automation development and implementation. With the PAK system, unit operation integration is mediated by system-monitored break vessels. For this run, this included the (1) harvest break vessel, (2) protein A capture product vessel, and (3) VI product flow kit vessel. The harvest break vessel and the virus inactivation neutralized product break vessel were controlled to a level setpoint using PID feedback control over the vessel outlet and inlet pump, respectively. The protein A product vessel used minimum, low, high, and maximum level criteria to trigger downstream pause/restart events. Overall, all vessels were controlled as intended. The significance of this control strategy is that it eliminates the concern of over/under flow of intermediate break vessels.

The protein A elution vessel changeover performed on day 9, as described in the Bioburden Control section, resulted in a buildup of cell culture material in the harvest vessel, as the system was paused during this time while perfusion material continued to accumulate. The 10L capacity of the harvest vessel coupled with its 50L overflow bag provided ample time for an operator to swap vessels and restart the system without further incident. The protein A product vessel triggered the downstream operations to begin once reaching a target level of 7.5 liters. After draining to a level of 1.5 liters, the downstream unit operations were triggered to pause to allow the vessel to refill. These trigger levels were manually changed through the HMI throughout the run to allow for the trigger events to coincide with an operator onsite. This was due to an abundance of caution and is not required for the process to operate.

A critical, yet often overlooked parameter in a continuous process is the mass flow rate of the overall process. Rather than set each unit operation to a specified flow rate and over-size break vessels to absorb differences, the PAK system actively controls the flow rate of each individual step and the system as a whole. This is achieved by controlling the last product pump in series to a flow rate set point, while regulating flow of upstream product pumps to maintain intermediate break vessel level (the protein A capture step is an exception). As a result of the vessel level control, any change in flow rate of the last product pump results

in a corresponding change in flow rate of the upstream product pumps. This control strategy allows for low break vessel volumes (150 ml for this 50 L cell culture per day process).

The mass flow rate set point (set for the last product pump in series) is determined by the most critical process parameter. For this run, the most critical process parameter was determined to be the virus inactivation residence time. Therefore, the mass flow rate of the entire process was controlled to ensure the VI residence time setpoint was met. A schematic of this process is displayed in Figure 7.

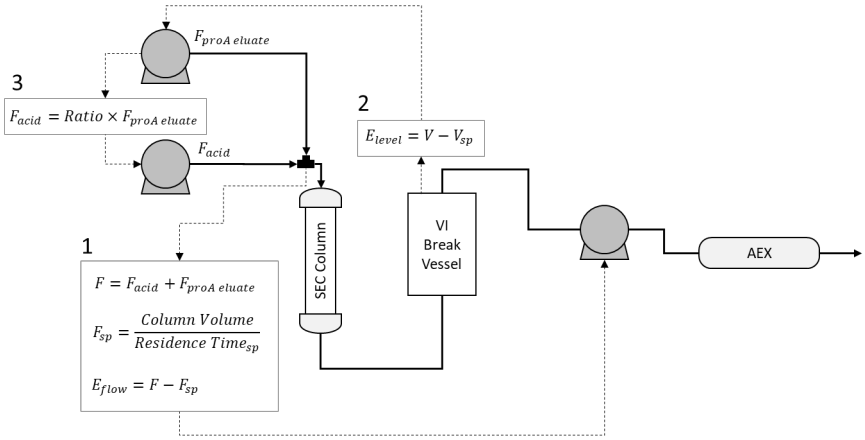


Figure 7. Schematic of mass flow rate control. (1) $F_{proAeluate}$: protein A product material flow rate, F_{acid}: acid flow rate, F: total flow into VI SEC column, F_{sp}: set point flow rate, E_{flow}: error in target flow into SEC column. (2) E_{level}: Error in target vessel level, V: vessel level, V_{sp}: set point vessel level. (3) Ratio: target ratio of acid flowrate to product flowrate.

The following steps are performed to adjust the pump flow rates to ensure the virus inactivation residence time set point is met. First, the total flow into the virus inactivation SEC column is calculated as the sum of the protein A product feed (VI product pump) and the VI acid pump flow rates. The flow rate set point that corresponds to the virus inactivation residence time target is then subtracted from the total inlet flow, providing the flow rate error. The flow rate error is fed into a PID controller that controls the AEX product pump (the last product pump in series in this process). Because this pump pulls from the VI break vessel, any change in this flow rate will immediately result in a change in VI product vessel level. The error of this vessel is fed into a PID controller that controls the VI product pump to maintain a constant VI vessel level. Finally, the VI acid pump output increases or decreases so that the target ratio between the VI acid and VI product pumps is always achieved.

The virus inactivation residence time for the third VI sub-run is displayed in Figure 5c.

This series of independent control loops, connected only by the physical properties of the VI product vessel allows for cohesive mass flow control across unit operations. Additional unit operations can be added between the VI and AEX steps with this strategy. In this case, flow control cascades up through multiple break vessels.

The perfusion process required a break in mass flow rate at some point in the process. The perfusion rate must be set constant and for this run the VI residence time was controlled to a setpoint. Regardless of the accuracy of pumps, there will inevitably be a difference in mass flow rates between these two unit operations. The break in mass flow rate occurred between the protein A chromatography step and the virus inactivation step. The difference in mass flow rate was absorbed by the protein A product break vessel. This is the reason the protein A product vessel operated under min, low, high, max control rather than continuous PID control and why the virus inactivation step ran as three separate sub batches during this run. In future runs the VI step will operate within a range of acceptable residence times which will allow VI flow rates to adjust

to match the changing output of the protein A eluate vessel. This will enable this step to run continuously for the duration of the process instead of periodically stopping and restarting.

Viral Clearance Data from continuous Virus Inactivation

To ensure viral clearance across the flow through virus inactivation SEC column, live virus spiking studies were performed. These studies aimed to (1) characterize the breakthrough profile of virus and (2) to ensure inactivation kinetics occur in the same manner to batch inactivation.

Before testing the column with the virus-spike, the breakthrough profile of the column was characterized with blue dextran as a virus surrogate. Blue dextran is a large molecule with a molecular weight of ˜2000kDa (˜10-20nm diameter). It is commonly used to determine column void volume and is expected to behave similarly to enveloped viruses, which are typically larger than 50 nm (ICH Q5A, Food and Drug Administration, 1999). Blue dextran spiking studies were performed at various flow rates across the SEC column to characterize the breakthrough profiles of different column residence times. Data can be seen in Figure 8.

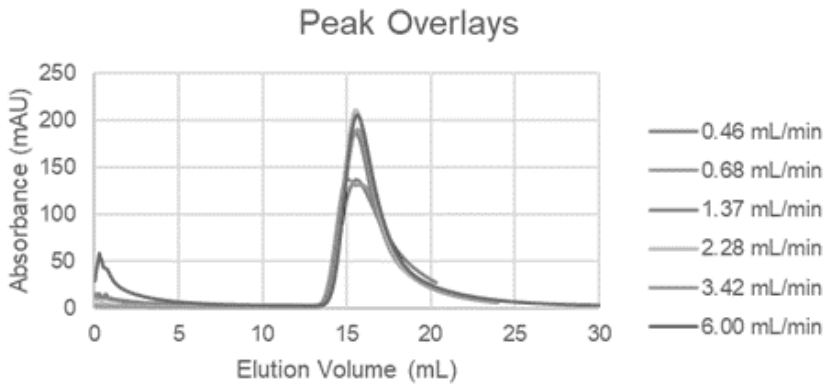


Figure 8. Peak overlays of all blue dextran elutions. Variance in peak height is due to variations in blue dextran spiking concentration and is irrelevant to the results.

As can be seen from the results, breakthrough volume was independent of flow rate, with a standard deviation of only 0.004 column volumes. This can be explained by the Van Deemter Equation:

$$HETP = A + \frac{B}{u} + (C_s + C_m) \bullet u$$

Where A is the constant eddy diffusion parameter, B is the diffusion coefficient in the longitudinal direction, and Cs and Cm are the resistance to mass transfer in the stationary and mobile phases, respectively. Because there is no mass transfer of blue dextran from the liquid phase to the solid phase or vice versa, this portion of the equation can be ignored. The flow regime is also operated at a relatively large u, making the longitudinal diffusion part of the equation negligible as well. This eliminates any dependence of velocity on peak broadening. These results are significant because they demonstrate a consistent breakthrough profile regardless of flow rate, which may need to be adjusted throughout the course of a continuous process. Therefore, breakthrough time across the column can be easily calculated from the current flow rate, column volume, and void volume coefficient. This is not necessarily true of other methods of flow through virus inactivation that rely on Dean vortices for axial mixing.

In order to demonstrate that live virus behaves similar to blue dextran, the column was spiked with inactivated xMuLV virus while running at a residence time of 30 minutes. Three 6 mL fractions of eluate were

collected corresponding to before, during, and after the expected breakthrough of virus. Results can be seen in Figure 9.

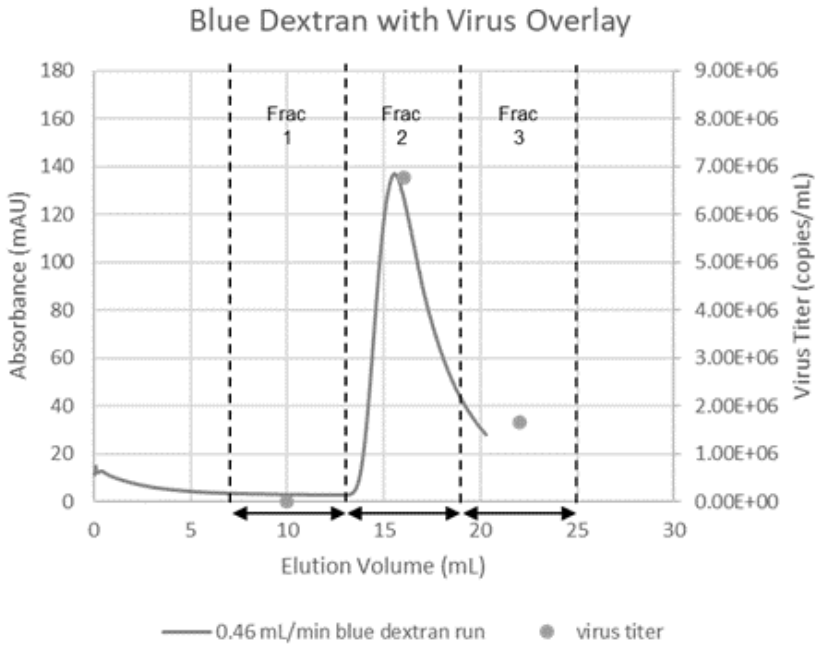


Figure 9. Breakthrough of XMuLV overlayed with a blue dextran run. Both datasets performed at 7.3cm/hr.

The pre breakthrough fraction contained $2.85x10^1$ virus copies/mL indicating little to no breakthrough as expected. The expected breakthrough fraction contained $6.78x10^6$ virus particles and the post breakthrough fraction contained $1.65x10^6$ virus particles. This follows a similar profile to the blue dextran elution profile, and most importantly shows virus breakthrough does not occur earlier than blue dextran.

In addition to breakthrough characterization, virus inactivation was also evaluated at multiple residence times across the column. Virus inactivation was measured at residence times of 2, 10, 20, and 30 minutes. Log inactivation data is shown in Figure 10.

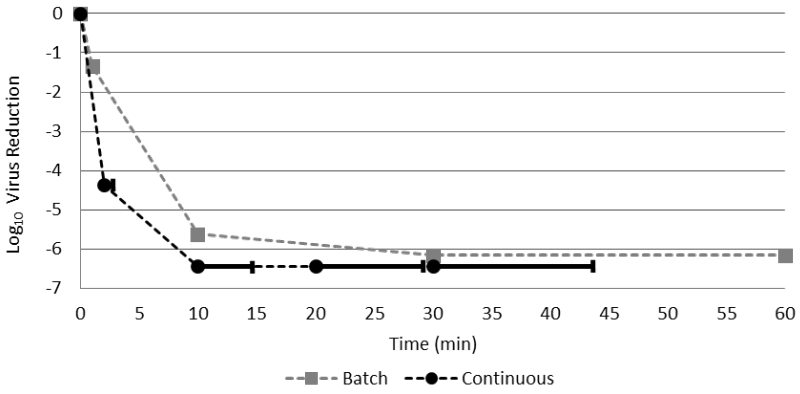


Figure 10. Log reduction values for both continuous and traditional batch modes of low pH virus inactivation. Both datasets performed at pH 3.6 with the same molecule. Dots on continuous dataset indicate start of collection of eluate sample while lines indicate the span of collection of the sample.

The log inactivation data shows a similar trend to the batch inactivation data for the same molecule under the same conditions. This clearly shows there is no difference in the kinetics of inactivation between batch and continuous methods.

Conclusion

We have successfully demonstrated a next generation continuous purification process for a 14-day operation with bioburden control. The PAK automated system for continuous purification provided the automation control, automation hardware and single-use assemblies required. Advanced control strategies controlled flow from one step to the next and the mass flow rate of the entire process. The control parameters were continuously monitored and set to alarm when limits were reached. No additional automation control or SCADA system was required to perform this work. Automation used was 21CFR Part 11 compliant and all product contact materials met or exceeded USP Class VI specifications. The Low pH continuous viral inactivation method used in this process was supported by off-line virus spiking studies and results are comparable to the batch process. Batch chromatography studies supported the dual column operations performed in the continuous process. Additional studies are required to confirm viral filtration efficacy at the reduced flow rates used in the continuous process. The technology presented enables implementation of continuous fully integrated processes at scales up to 200L of cell culture material per day, which is a significant step towards good manufacturing practice (GMP) implementation of end-to-end continuous purification processes.

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